

Growth and Sporulation Potential of *Clostridium perfringens* in Aerobic and Vacuum-Packaged Cooked Beef

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ABSTRACT

Growth of *Clostridium perfringens* in aerobic- and anaerobic- (vacuum) packaged cooked ground beef was investigated. Autoclaved ground beef was inoculated with $\sim 3.0 \cdot \log_{10}$ CFU/g of *C. perfringens*, packaged and stored at various temperatures. Vegetative cells and heat-resistant spores were enumerated by plating unheated and heated (75°C for 20 min) meat samples on tryptose-sulfite-cycloserine agar. *Clostridium perfringens* grew to >7 logs within 12 h at 28, 37 and 42°C under anaerobic atmosphere and at 37 and 42°C under aerobic conditions. At 28°C under aerobic conditions, growth was relatively slow and total viable count increased to >6 logs within 36 h. Similarly, growth at 15°C in air was both slower and less than under vacuum. Regardless of packaging, the organism either declined or did not grow at 4, 8 and 12°C. Spores were not found at $<12^\circ\text{C}$. Spores were detected as early as 8 h at 42°C under anaerobic conditions, but in general, the type of atmosphere had little influence on sporulation at $\geq 28^\circ\text{C}$. Temperature abuse (28°C storage) of refrigerated products for 6 h will not permit *C. perfringens* growth. However, cyclic and static temperature abuse of such products for relatively long periods may lead to high and dangerous numbers of organisms. Reheating such products to an internal temperature of 65°C before consumption would prevent food poisoning since the vegetative cells were killed.

Clostridium perfringens is responsible for one of the most common types of food poisoning worldwide (5,7,15). Outbreaks primarily involve meat and meat products. In the 15-year period, 1973-1987, beef was the vehicle for 26.8% of the *C. perfringens* outbreaks (51/190); the organism accounted for 10.2% of the total bacterial foodborne outbreaks (190/1869) and 11.2% of the total cases of bacterial foodborne disease (12,234/108,806) in the United States (2). Todd (16,17) estimated that 652,000 cases of foodborne *C. perfringens* illness occur each year in the United States with an average of 7.6 deaths and annual costs of \$123 million.

Precooked, vacuum-packaged, uncured refrigerated beef products are increasingly common in U.S. markets. Normal heat treatments given to these products are insufficient to inactivate *C. perfringens* spores. Also, these products may

be contaminated with spores or vegetative cells after cooking. Food poisoning by *C. perfringens* has been traditionally associated with temperature abuse in institutional food service settings. The trend in the food industry, however, to market ready-to-eat entrees raises the possibility for outbreaks in supermarket and home prepared foods. The importance of *C. perfringens* is increased by reports that some strains can grow at 6°C (11). Though not a true psychrophile, this microorganism can grow at common refrigeration temperatures, thought previously to inhibit or prevent the growth of foodborne pathogens.

In a study by Hintlian and Hotchkiss (10), when cooked, roast beef coinoculated with *C. perfringens* and *Pseudomonas fragi* was packaged under modified atmosphere (75% carbon dioxide, remainder oxygen) and stored at 12.8 or 26.7°C, a combination of modified atmosphere and low temperature (12.8°C) was reported to inhibit *C. perfringens* growth. While the study by Hintlian and Hotchkiss (10) provided some characterization of *C. perfringens* in modified atmospheres, there appears to be no work available on growth in vacuum-packaged beef. Accordingly, the objectives of the current study were to investigate (i) the potential for growth and sporulation of *C. perfringens* in precooked, aerobic- and vacuum-packaged beef stored at refrigerated temperatures, (ii) the effect of temperature abuse (static and cyclic), and (iii) the effect of reheating of the refrigerated and temperature abused meat samples on the fate of this organism.

MATERIALS AND METHODS

Test organism

Clostridium perfringens strain NCTC 8239 (Hobbs serotype 3) obtained from American Type Culture Collection was used in the study. The organism was maintained at 4°C in cooked-meat medium (Difco Laboratories, Detroit, MI) and used through the course of the study as the stock culture.

Preparation of inoculum

To prepare inocula, 0.1 ml from the stock culture was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (FTM). The inoculated medium was then heat shocked at 75°C for 20 min and incubated at 37°C for 18 h. The cells were harvested by centrifugation at room temperature for 10 min at

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7,700 × g, the cell pellet washed twice and finally resuspended in sterile 0.1% peptone water (wt/vol).

Sample preparation and inoculation

Ground beef was obtained from a local retail market and frozen (-5°C) until use (approximately 40 days). After thawing, the meat was placed in a thin layer on plastic trays and autoclaved at 121°C for 15 min. The fat was poured off while the beef was hot and the meat cooled at 4°C to an internal temperature of 25°C. The pH of the ground beef was determined using a combination electrode (Sensorex, semi-micro, A. H. Thomas, Philadelphia, PA) attached to an Orion Model 601A pH meter. Duplicate 25-g ground beef samples were aseptically weighed into filter stomacher bags (SFB-0410; Spiral Biotech., Bethesda, MD) and inoculated with 1 ml of *C. perfringens* cell suspension so that the final concentration of cells was approximately \log_{10} 3 CFU/g. Thereafter, the bags were manually mixed to ensure even distribution of the organisms in the meat sample. Half of the bags were placed in 7" × 8" plastic barrier bags (Koch Model 01 46 09, Kansas City, MO). The oxygen transmission rate of the nylon/polyethylene film was 3.5 cc/100 in² in 24 h measured at 75°F and 75% relative humidity. The bags were evacuated to a negative pressure of 1,000 millibars and heat sealed using a Multivac Model A300/16 gas packaging machine (Germany). Two replications were performed for each atmospheric treatment.

Storage, temperature abuse, and sampling

The inoculated aerobic- and vacuum-packaged samples were stored at 4, 8, 12, 15, 28, 37 and 42°C. Samples from each atmosphere and storage temperature ≤15°C were analyzed on day 4, 8, 16, 24, 32 and 40. Samples stored at ≥28°C were analyzed at 4, 8, 12, 24, 36 and 48 h. To determine the effect of cyclic and static temperature abuse, samples stored at 4°C were moved 4 days before their scheduled sampling day (4, 8, 16, 24, 32 and 40) to 28°C, held at this temperature for 24 h, and then returned to 4°C and plated on their scheduled sampling day. Some samples were transferred to 28°C for 6 and 20 h before plating on their scheduled sampling day.

Bacterial enumeration

On the scheduled sampling day, samples were removed and enumerated for vegetative growth and heat-resistant spores by spiral plating (Spiral Systems Model D plating instruments; Cincinnati, OH). Sterile 0.1% peptone water (25 ml) was added to each bag to give a 1:1 (wt/vol) slurry and homogenized for 1 min with a stomacher Lab-blender (Model 400, Spiral Systems, Inc.). A 5-ml portion of the slurry was heat shocked for 20 min at 75°C to kill vegetative cells and to activate heat-resistant spores for spore quantitation. Vegetative cell counts were determined from unheated portions. Serial dilutions of heat and nonheat-shocked samples were made in 0.1% peptone water (wt/vol) followed by spiral plating of each dilution in duplicate on tryptose-sulfite-cycloserine (TSC) agar without egg yolk enrichment (9). The TSC agar plates were overlaid with an additional 10 ml of TSC agar. After overlaying, the plates were allowed to solidify before placing into anaerobic jars. Vegetative cells and viable spore counts were determined after 48 h of incubation at 37°C in a GasPak system (Baltimore Biological Laboratory, Cockeysville, MD).

Reheating and holding temperature

To determine the fate of *C. perfringens* vegetative cells and spores surviving reheating temperature, 25 g of ground beef samples inoculated with 3×10^6 CFU/g were vacuum-packaged and stored at 4°C for 7 days and at 28°C for 36 h. Thereafter, two bags from each incubation temperature (4 and 28°C) were opened and a sterile copper-constantan thermocouple was placed at the

center of the ground beef. The bags were sealed under vacuum and submerged in a 65 or 98°C operating water bath (Exacal, Model EX-251HT, NESLAB Instruments, Inc., Newington, NH) along with the experimental samples. The internal temperature of the samples was constantly monitored by the thermocouples. The readings were measured and recorded by a Keithly-Metabyte data logger connected to a microcomputer. The thermocouple signal was sampled every second, and the two readings were averaged to determine the sample temperature. Samples were removed when the internal temperature reached the target temperatures of 45, 55, 65, 75, 85 and 95°C. After reheating, packages were opened and incubated aerobically at room temperature. Samples were analyzed for *C. perfringens* vegetative cells and spores at 1.5, 3, 4.5, 6, 12 and 24 h.

Data processing

Bacterial growth curves were generated from the experimental data using the Gompertz equation (8) in conjunction with ABACUS, a nonlinear regression program that employs a Gauss-Newton iteration procedure. This FORTRAN-based program was developed by W. C. Damert (Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, PA). The Gompertz parameter values were subsequently used to calculate generation times and lag times as described by Gibson et al. (8).

RESULTS AND DISCUSSION

The pH of the cooked ground beef used in the study was 6.25. *C. perfringens* grew rapidly in aerobic- and anaerobic (vacuum)-packaged precooked ground beef during storage at 28°C and above (Fig. 1). Mean \log_{10} CFU/g after 12 h at ≥28°C increased from 3.02 to >7 for vacuum-storage samples and 2.61 to >6 within 12 h for air-storage samples at 37 and 42°C. At 28°C, the growth under aerobic conditions was relatively slow taking 36 h to increase to >6 \log_{10} CFU/g. Naik and Duncan (12) reported that in moist cooked ground beef stored at 37°C, total viable counts of *C. perfringens* increased from 4.74- to 7.34- \log_{10} CFU/g aerobically and from 5.30- to 7.48- \log_{10} CFU/g anaerobically in 4 h and remained relatively constant at approximately 8 logs for 24 h. In the present study, the generation time under aerobic conditions was 17.4, 14.8 and 103.8 min at 42, 37 and 28°C, respectively. Anaerobically, the generation times were 21.6, 21.6 and 118.9 min at 42, 37 and 28°C (Table 1). The lag times were shorter for anaerobically grown cells by approximately 2 h at 37 or 42°C as compared to aerobically grown cells. *C. perfringens* exhibited a 5 × shorter lag time under vacuum at 15°C (Table 1). Willardsen et al. (18) reported that *C. perfringens* strain NCTC 8238 grew rapidly and exhibited a generation time of 17.3 and 7.1 min at 33 and 41°C in autoclaved ground beef stored aerobically. In the same study, a composite of various strains of *C. perfringens* vegetative cells in autoclaved ground beef exhibited generation times of 39.4, 19.4 and 8.9 min at 26, 33 and 41°C, respectively. In another study, generation time and lag time of a *C. perfringens* 8-strain composite in autoclaved ground beef under air at 41°C was 8.8 min and 1.3 h, respectively (19). In general, the longer generation times observed in our study may be attributed to strain variation.

At 15°C, the type of atmosphere dramatically influenced the growth of *C. perfringens* with little or no growth for 25 days aerobically and only a 1.5-log increase in 40

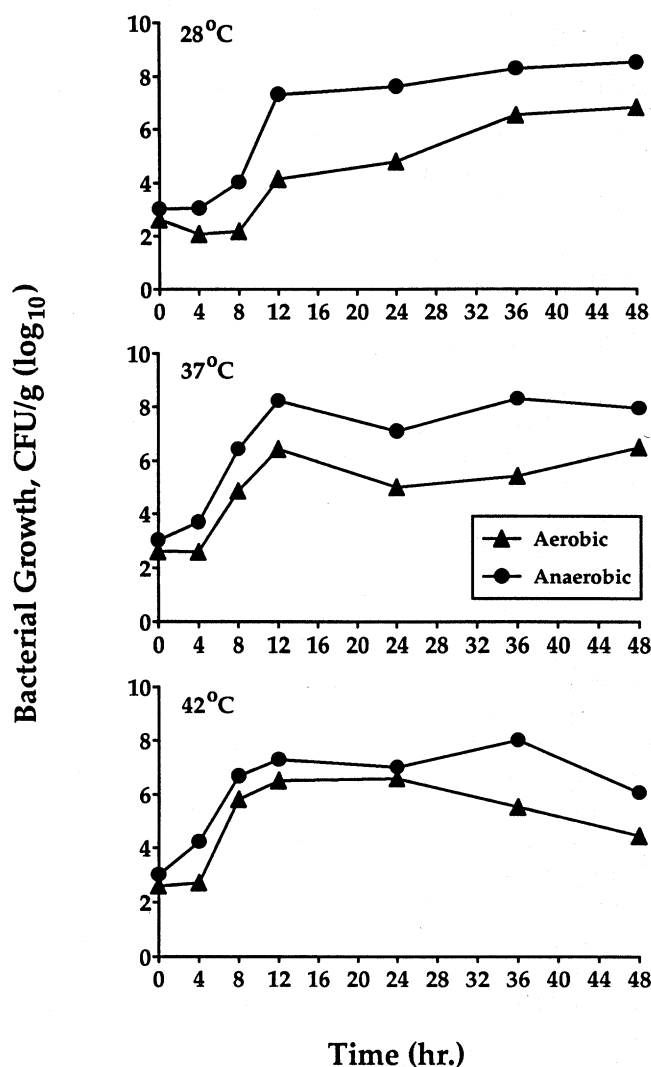


Figure 1. The effect of temperature abuse on growth of *C. perfringens* at 28, 37 and 42°C in aerobic and vacuum-packaged cooked ground beef.

TABLE 1. Mean generation times (G_i) and lag times^a of *C. perfringens* in autoclaved ground beef at various temperatures

Temperature (°C)	Aerobic		Anaerobic	
	G_i (min) ^b	Lag (h)	G_i (min)	Lag (h)
42	17.4	4.36	21.6	2.56
37	14.8	6.12	21.6	3.50
28	103.8	7.92	118.9	7.06
15	2652.0	585.60	2148.0	112.56

^a Means represent two replications.

^b Generation times calculated from regression lines for exponential growth using the Gompertz equation.

days (Fig. 2). Under vacuum, there was a 3-log increase at 16 days and 5 logs at 40 days (Fig. 2).

There was a decline in population densities from 2.61-2.99-log₁₀ CFU/g at day 0 to 1.01-1.32-log₁₀ CFU/g after 40 days under both atmospheres at 8 and 12°C (Fig. 2). Similar declines were seen in 4°C storage under both atmospheres at 40 days (Fig. 3).

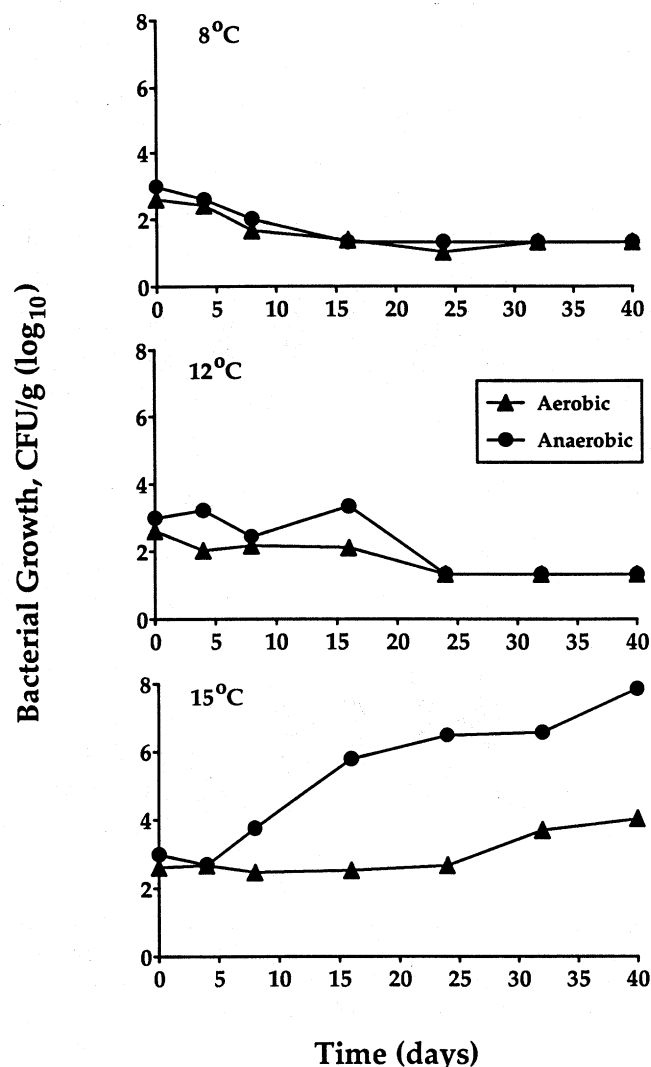


Figure 2. The effect of temperature abuse on growth of *C. perfringens* at 8, 12 and 15°C in aerobic and vacuum-packaged cooked ground beef.

To simulate the cyclic temperature abuse of refrigerated meat products which may occur in supermarkets, ground meat samples were transferred to 28°C for 24 h, 4 days before their scheduled sampling day. The numbers of the organism increased to >6 logs regardless of the packaging or further length of storage at 4°C (Fig. 3). When aerobically and anaerobically packaged samples were temperature abused to 28°C for 6 h before plating on their scheduled sampling day, the total viable count in all meat samples were <3 logs (data not shown). Thus, a short period of abuse does not foster the growth of *C. perfringens*. However, temperature abuse to 28°C for 20 h before plating resulted in increase to >6 logs of CFU/g in all samples (Table 2). Additional studies indicated no increase in growth for cyclic and static temperature abuse of ≤10 h.

While *C. perfringens* did not sporulate at <12°C, 1.68-log₁₀ spores per g were detected by day 16 at 15°C only in vacuum-packaged beef (Table 3). The type of atmosphere had little influence on sporulation at 28°C and above; however, 2.84-log₁₀ spores per gram were detected as early as 8 h at 42°C under anaerobic conditions. Spore counts

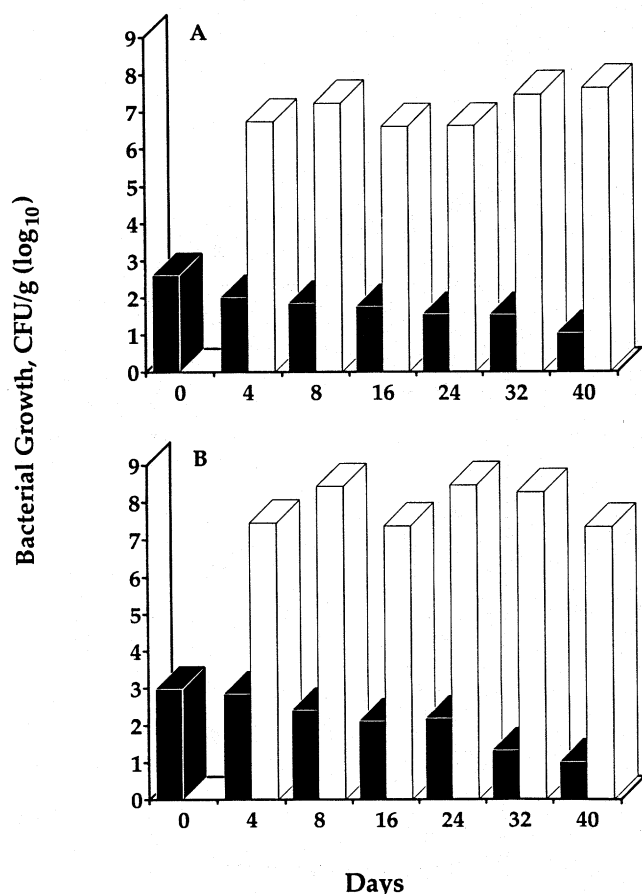


Figure 3. The effect of cyclic temperature abuse on growth of *C. perfringens* in cooked ground beef stored at 4°C (■). The samples were moved to 28°C for 24 h, 4 days before their scheduled sampling day (□). (A) aerobic and (B) vacuum-packaged.

TABLE 2. The effect of temperature abuse on growth of *C. perfringens* in anaerobically and aerobically packaged cooked ground beef stored at 4°C.

Atmosphere	Followed by hours at		Count ^{a,b}
	Days at 4°C	28°C	
Aerobic	40	-	1.04
	7	20	7.29
	15	20	8.13
	23	20	7.57
	31	20	8.38
	39	20	7.24
Anaerobic	40	-	1.01
	7	20	7.78
	15	20	7.95
	23	20	6.38
	31	20	8.13
	39	20	7.47

^a Starting count about 2.75-log₁₀ CFU/ml.

^b Data represent means of two replications.

ranged from 1.68- to 4.53-log₁₀ CFU/g anaerobically at temperatures ranging from 15 to 42°C, whereas sporulation ranged from 1.32- to 2.02-log₁₀ spores per gram aerobically

TABLE 3. Earliest time when *C. perfringens* spores were detected in anaerobically and aerobically packaged cooked ground beef stored at different temperatures.

Temperature	Storage conditions	Time to spore detection ^{a,b} (h)
42°C	Anaerobic	8 (2.84)
	Aerobic	24 (1.32)
37°C	Anaerobic	36 (2.81)
	Aerobic	24 (1.32)
28°C	Anaerobic	36 (4.53)
	Aerobic	24 (2.02)
15°C	Anaerobic	384 (1.68)
	Aerobic	ND ^c

^a Values in parentheses indicate the count in log₁₀ CFU/g.

^b Data represent means of two replications.

^c Not detected.

at ≥28°C. Naik and Duncan (12) reported 4.20-log₁₀ spores per gram in moist cooked ground beef inoculated with about 5-log₁₀ CFU/g and stored aerobically at 37°C in 24 h, while anaerobically, it required only 12 h to reach 4.11-log₁₀ spores per g. However, at room temperature, the spore count increased to 3.23-log₁₀ spores per gram at 24 h anaerobically, while the levels were less than 30 spores in samples incubated aerobically (12).

In precooked, vacuum-packaged, uncured refrigerated beef products, inadequate reheating temperatures may contribute to *C. perfringens* food poisoning. In fact, reheating of precooked foods before consumption may eliminate vegetative cells and heat shock spores into activation and germination. For example, Pirie and Harrigan (13) reported that sublethal heat treatment of 87.7 to 90°C for 3 min in precooking meat preparations for meat pies apparently induced germination of spores that were later killed by further heating at 89°C after a cooling period of 30 min. In the present study, when vacuum-packaged samples were temperature abused at 28°C for 36 h (to induce spore formation) and then subsequently reheated in a water bath to an internal temperature of 65°C and above, vegetative cells were inactivated and 2- to 3-log₁₀ spores per gram were recovered (Table 4). The spore levels remained in the order of 2 to 3 logs even after 24 h of storage at room temperature, but no spore germination occurred (Table 4). This may be attributed to the fact that *C. perfringens* spores were not heat shocked during reheating. Heat treatment for 20 min at 75°C is required to heat shock the spores with resultant germination and outgrowth (11). Craven (6) reported that spores will germinate at a reduced rate without prior heat shock. In a study by Barnes et al. (1), about 3% of spores germinated in raw beef without prior heat shock, but almost all germinated after the meat was heated. Reheating the ground beef to an internal temperature of ≤55°C did not reduce the total viable cell count, and the cell number remained at about 7-log₁₀ CFU/g at all sampling times. Reheating the samples stored at 4°C for 7 d to an internal temperature of 65°C killed all vegetative cells (Table 5). However, exposure of packages to an internal temperature of 45 or 55°C did not reduce the *C. perfringens* population (Table 5). There was no multiplication of the

TABLE 4. Effect of reheating temperature (45-95°C) and subsequent room temperature holding of precooked vacuum-packaged temperature abused ground beef^a on the fate of *C. perfringens* vegetative cells and spores^{b,c}.

Holding time at (h) 25°C	Reheating temperature (°C)					
	45	55	65	75	85	95
0	7.70	7.75	3.27	3.22	3.16	2.44
1.5	7.70	7.77	3.24	3.16	3.01	2.32
3	7.78	7.78	3.19	3.29	3.16	2.32
4.5	7.88	7.85	3.18	3.30	3.18	2.32
6	7.92	7.88	3.04	3.09	3.13	2.47
12	6.41	6.63	3.19	3.27	3.16	2.32
24	6.78	6.83	3.94	3.17	2.70	2.32

^a Autoclaved ground beef inoculated with about \log_{10} 3 CFU/g was vacuum packaged and stored at 28°C for 36 h.

^b Initial number of vegetative cells were about 7 logs and spores about 3 logs/g.

^c Each count represents means of two replications and expressed as \log_{10} CFU/g.

TABLE 5. Effect of reheating temperature (45 to 65°C) and room temperature holding of precooked vacuum-packaged refrigerated ground beef^a on the fate of *C. perfringens* vegetative cells^{b,c}.

Holding time at 25°C (h)	Reheating temperature (°C)		
	45	55	65
0	2.55	2.62	ND ^d
1.5	2.55	2.62	ND
3	2.47	2.55	ND
4.5	2.85	2.97	ND
6	3.06	3.10	ND
12	3.16	3.16	ND
24	2.70	2.62	ND

^a Autoclaved ground beef inoculated with about $3\text{-}\log_{10}$ CFU/g was vacuum packaged and stored at 4°C for 7 days.

^b Initial number of vegetative cells was about $3\text{-}\log_{10}$ CFU/g.

^c Each count represents means of two replications and expressed as \log_{10} CFU/g.

^d Not detected.

cells during storage at room temperature for 24 h following the sublethal heat treatment which may have injured the vegetative cells. Willardsen et al. (18) reported that inactivation of *C. perfringens* in ground beef began at approximately 55°C in a constantly rising temperature (rate 8.5°C/h) water bath, but a minimal exposure to temperatures near 60°C may be insufficient to inactivate vegetative cells of *C. perfringens*. Smith et al. (14) found that cooking beef in a water bath to an internal temperature of 60°C and holding for at least 12 min reduced a population of *C. perfringens* by approximately 3-log cycles. Bryan (3) suggested a minimum temperature of 74°C for reheating meat, but he later revised the internal temperature to 70°C (4). In a study by Wright-Rudolph et al. (20), when ground beef patties inoculated with 5 log/g cells were cooked to an internal temperature ranging from 65 to 70°C (rare) to 77 to 93°C

(well done), reduction in counts ranged from 0.75 to 1.48 \log_{10} /g during microwave cooking and from 3.51 to 8.06 \log_{10} /g for conventional cooking.

Smith et al. (14) indicated that the heat transfer coefficient between the cooking medium and the meat is roughly 20 times greater when the product is cooked in water as opposed to air. Therefore, transfer of heat is faster when the product is cooked in a water bath, and there is a greater potential for microbiologically safe food.

It was demonstrated that proper refrigeration will prevent multiplication of the organism, and that the population stabilizes or slowly decreases. *C. perfringens* may grow to possible infective levels of at least $5\text{-}\log_{10}$ CFU/g if precooked, vacuum-packaged, uncured, refrigerated beef products are poorly handled or temperature abused for a relatively long period. Abused precooked foods, if not reheated adequately before consumption, would be a potential source for food poisoning. However, our results indicated that reheating to 65°C would be sufficient to inactivate *C. perfringens* vegetative cells.

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